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Early Development in the Lancelet (=Amphioxus) *Branchiostoma floridae* from Sperm Entry through Pronuclear Fusion: Presence of Vegetal Pole Plasm and Lack of Conspicuous Ooplasmic Segregation

LINDA Z. HOLLAND AND NICHOLAS D. HOLLAND

Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093-0202

Abstract. Lancelet eggs are described from serial fine sections before fertilization and at frequent intervals thereafter until the male and female pronuclei meet at 16 min after insemination. In the unfertilized egg, although mitochondria, as well as yolk granules, are evenly distributed (both are absent only from the egg cortex and meiotic spindle), the mitochondria in the animal third have a more electron-lucent matrix than those elsewhere. The cortex of the unfertilized egg is occupied chiefly by cortical granules, and the subcortical cytoplasm in the vegetal third includes sheets of dense granules interleaved with cisternae of endoplasmic reticulum. By 45 s after insemination, (1) the fertilizing sperm enters (in the animal hemisphere in three out of three observations), (2) yolk granules become patchily distributed around the newly entered sperm, (3) cortical granule exocytosis occurs, and (4) the sheets of dense granules and associated endoplasmic reticulum aggregate with numerous mitochondria into whorls in a yolk-free zone near the vegetal pole. These whorls are the vegetal pole plasm, which is segregated into a single blastomere at each cleavage and might play a role in germ line determination. By 2 min after insemination, the zone of cytoplasm near the animal pole with patchily distributed yolk has enlarged, and the male pronucleus has migrated to the vicinity of the vegetal pole and formed an aster, at the center of which a few mitochondria are aggregated. In lancelets, unlike ascidians, there is no obvious widespread ooplasmic segregation or translocation of cytoplasm from animal to vegetal pole accompanying the movement of the sperm. Between 6 and 16 min, (1) the zone of cytoplasm with patchily distributed yolk enlarges to occupy about the animal third of the egg, (2) the female pronucleus forms by fusion of chromosome-containing vesicles and migrates vegetally, leaving a track of yolk-poor cytoplasm, and (3) the male pronucleus, surrounded by increasing numbers of mitochondria, migrates to meet the female pronucleus just above the equator. In contrast to current opinion, lancelets differ from ascidians both in having a vegetal pole plasm and in lacking marked ooplasmic segregation.

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Introduction

The importance of lancelets in chordate evolution was first revealed by the embryological studies of Kowalevsky (1865, 1867). His work stimulated many descriptive studies on the development of lancelets aimed at clarifying their phylogenetic relations (*e.g.*, Hatschek, 1882, 1893; Lwoff, 1892; van Wijhe, 1893; Willey, 1894). In the first experimental study of this problem, Wilson (1893) investigated the development of isolated blastomeres and partial embryos. He concluded that the regulative capacity of lancelets is intermediate between that of echinoids and ascidians; that is, blastomere fates become determined in ascidians, lancelets, and echinoids by the second, third, and fourth cleavages, respectively.

Until recently, studies of lancelet embryology could deal only superficially with events before first cleavage because of difficulty in obtaining the earliest stages: artificial fertilization was never achieved, and, therefore, embryos were collected after males and females had spawned together in the field or the laboratory. Thus, descriptions of early events like the cortical reaction, pronuclear move-

ments, and maturation divisions (van der Stricht, 1896; Sobotta, 1895, 1897; Cerfontaine, 1906) were based on relatively incomplete material.

Conklin (1932, 1933) reinvestigated both the descriptive and the experimental studies with special attention to possible similarities between lancelets and ascidians. He had already established his reputation as an authority on protochordate development with two papers (1905a, b) on the embryology of the ascidian *Styela partita*. His descriptive work (1905a) showed that ooplasmic rearrangements between fertilization and pronuclear fusion in *S. partita* segregated the following five areas of cytoplasm destined to be incorporated into specific embryonic tissues: (1) the yolk-poor, dark yellow myoplasm with abundant mitochondria and pigment granules, destined for the larval tail muscles, (2) an adjacent light yellow mesenchyme material (the myoplasm and mesenchyme together comprise the mesodermal crescent), (3) the yolk-poor, clear ectoplasm, the precursor of the ectoderm, (4) the yolk-rich, dark grey endoplasm, that becomes the endoderm, and (5) a light grey cytoplasm destined for the notochord and neural plate. In his experimental work, Conklin (1905b) reported that individual blastomeres and groups of blastomeres separated at or beyond the second cleavage and reared in isolation had the same developmental fate as in the intact embryo. Thus, he concluded that the ooplasmic segregation created a mosaic of organ-forming substances in the ascidian embryo and determined the fate of each region of the uncleaved, fertilized egg.

In 1910, Conklin began to study lancelet development, but had difficulty obtaining embryos. During the next 22 years, he obtained some additional material, but still lacked the earliest stages. Therefore, when Conklin finally published on lancelet embryology, he was forced to rely on van der Stricht (1896), Sobotta (1897), and Cerfontaine (1906) for all events before first cleavage. Nevertheless, Conklin concluded that in regard to pronuclear movements and ooplasmic segregation lancelets were "precisely like ascidians" (1932) and that "the localizations of materials in the Amphioxus egg are like those of ascidians, although not so sharply differentiated" (1933). In other words, the fate maps of ascidians and lancelets were identical. Furthermore, in contrast to Wilson (1893), Conklin (1933) believed that ooplasmic segregation ensured that "all axes and poles of the future larva are irreversibly determined at or before the first cleavage . . . ," and he concluded that "development in Amphioxus, as also in Ascidians, is a mosaic work." This conclusion has been widely accepted by later biologists (*e.g.*, Brien and Dalcq, 1948; Drach, 1948; Wall, 1990).

It has generally been overlooked that Tung *et al.* (1958, 1960a, b, 1962a, b) repeated and extended Conklin's experiments on lancelet embryos. They made some changes in Conklin's fate map, finding in particular that the distribution of mesodermal material is rather different from that of ascidians, being more like that of amphibians. In addition, Tung *et al.* (1958) supported the view of Wilson (1893) that the lancelet egg has a considerable regulative capacity, and they concluded that "the development of the egg of Amphioxus is, therefore, not a mosaic work as suggested by Conklin."

In light of the work of Tung *et al.* (1958), a reinvestigation with transmission electron microscopy (TEM) of Conklin's descriptive work on early embryology of lancelets is especially important. A recent TEM study of lancelet development by Hirakow and Kajita (1990) relied on natural spawnings and thus included only limited observations on fertilized, uncleaved eggs. This obstacle has recently been overcome with the development of methods for spawning and artificially fertilizing lancelet eggs (Holland and Holland, 1989a). In our initial study on the cortical reaction of *Branchiostoma floridae,* we showed conclusively, that unlike ascidian eggs, which lack cortical granules, lancelet eggs have cortical granules that undergo exocytosis at fertilization and contribute to the formation of the fertilization envelope (Holland and Holland, 1989a). In the present work, we extend our fine structural investigations to cover events between sperm entry and pronuclear fusion. We have followed the formation of the pronuclei and pronuclear migrations and have discovered a conspicuous vegetal pole plasm, but we have found no evidence for extensive ooplasmic segregation of the ascidian type.

Materials and Methods

Specimens of *Branchiostoma floridae* were collected in late summer of 1988, 1989, and 1990 in Old Tampa Bay, Florida. Spawning of females was induced by electrical shock, and sperm motility was stimulated by 10 m*M* NH_4Cl as previously described (Holland and Holland, 1989a). Because only a few of the sperm bound to eggs undergo the acrosome reaction, it was necessary to use a concentrated sperm suspension (roughly 1:500 to 1:1000 dilution of dry sperm) to obtain synchronous fertilization. Development was at 24°C; at that temperature, first cleavage occurs about 30 min after insemination, and gastrulation begins at about 5 h.

For TEM, eggs were fixed in 1% $K_2Cr_2O_7$, 3% glutaraldehyde, 0.7 *M* NaCl pH 7.4, and postfixed in the same buffer plus 1% OsO_4 and 0.7 *M* NaCl (Holland, 1988). For low-power TEM, some unfertilized eggs and some at 45 s after insemination were fixed as above with the NaCl lowered to 0.45 *M* to prevent the shrinkage that occurred before completion of the cortical reaction in eggs fixed in higher tonicity. Eggs were dehydrated in an ethanol series and embedded in Spurr's resin. This method, chosen be-

cause of good preservation of organelles, does not preserve some constituents of the chromosomes, which thus have a low electron density. We fixed unfertilized eggs as well as fertilized eggs at 15-s intervals up to 1.5 min after insemination, at 30-s intervals up to 2 min after insemination, at 1-min intervals up to 10 min after insemination, and at 2-min intervals to 32 min after insemination, the time of first cleavage. To determine the timing of pronuclear movements, serial 1–2 μm sections were stained with 1% toluidine blue in sodium borate and examined by light microscopy (LM). In the light of those results, one to five eggs were serially fine-sectioned at each of the following intervals after insemination: 0 s, 45 s, 2 min, 6 min, 10 min, and 16 min.

Results

Unfertilized egg (Figs. 1, 2)

The spawned, unfertilized egg of *Branchiostoma floridae* is about 140 μm in diameter and is arrested in metaphase of the second meiotic division. The animal pole is marked externally by the first polar body and internally by the second meiotic spindle (Figs. 1; 2A, B). Surrounding the egg and overlying the first polar body is a vitelline layer (Figs. 1; 2A, C; 3D). The egg cytoplasm contains a peripheral layer of cortical granules, which are closely apposed to one another except where the meiotic spindle intervenes (Figs. 1; 2B, C). During the first minute after insemination, the cortical granules undergo exocytosis, initiating elevation of a fertilization envelope as previously described (Holland and Holland, 1989a). The first polar body, of both unfertilized and fertilized eggs (Fig. 3D), typically includes some unreacted cortical granules and a cluster of chromosomes; a nucleus is lacking, although there are frequently a few profiles of nuclear envelope (Fig. 4B).

Within the egg cytoplasm, yolk granules, 2–5 μm in diameter, have a relatively even distribution, being excluded only from the cortical cytoplasm and the meiotic spindle (Fig. 2A). In about a 30° arc near the vegetal pole, just interior to the cortical granules, are several sheets of dense granular material stacked 2–4 deep, parallel to the egg plasma membrane (Figs. 1; 2C, D). These sheets are usually, but not always, interleaved with sheets of smooth endoplasmic reticulum (SER) (Fig. 2D), which are rare elsewhere in the cytoplasm. Although the granules comprising the sheets are similar in size to ribosomes, the arrangement of the granules and ER differs from that of rough ER: between two cisternae of SER there is only one sheet of granules, and it is separated from the SER on each side by a space 50 to 75 nm wide. Some mitochondria are usually situated near the sheets, but not in conspicuously greater abundance than elsewhere in the cytoplasm where they are fairly uniformly distributed (approximately 30/100 μm^3).

Although all the mitochondria in the unfertilized egg are about the same size (0.5 × up to 3 μm), those in a zone about 35 μm deep around the meiotic spindle have an electron-lucent matrix (Fig. 2E), while those elsewhere have a much denser matrix (Fig. 2D). This difference does not appear to be a fixation artifact since there is a narrow transition zone where both types of mitochondria co-occur (Fig. 5C). The distribution of the two types was similar in all eggs examined and did not change after fertilization, at least up until formation of the zygote nucleus.

Sperm entry: 30–45 s after insemination (Figs. 1, 3, 4)

Sperm of *Branchiostoma floridae* have a compact nucleus about 1.5 μm in diameter, a midpiece (with two centrioles and one mitochondrion), and a cup-shaped acrosome that can undergo an acrosome reaction producing a short acrosomal tubule (Holland and Holland, 1989b; unpub.). To determine the timing of sperm entry, 1–2 μm sections of eggs fixed at 15-s intervals after insemination were examined by light microscopy (LM). In two eggs fixed at 30 s after insemination, at the beginning of the cortical reaction, a sperm was seen by LM attached to the egg surface in the animal hemisphere via a short fertilization cone (data not shown). However, since condensed sperm nuclei are about the same density and size as yolk granules, no sperm nucleus could subsequently be detected in 1 μm sections until about 4 min after insemination, when it appeared as a clear sphere about 5 μm in diameter in a small yolk-free zone of cytoplasm near the vegetal pole. Fluorescent DNA-binding dyes also failed to reveal the newly entered sperm because its nucleus could not be differentiated from those of non-fertilizing sperm bound to the fertilization envelope. A large excess of sperm is required for synchronous fertilization, and many remain associated with the fertilization envelope, both at the animal pole and elsewhere, even after the cortical reaction (Fig. 3A, B, D). Therefore, to detect the fertilizing sperm just after entry, serial TEM sections were made through an egg at 45 s after insemination.

Two serial fine sections from the same egg at 45 s after insemination are shown in Figure 3A, B. The section in Figure 3A approximately bisects the egg, and the first polar body marks the animal pole at the top of the figure. Figure 3B, which is in the same orientation, but about half-way between the center and the edge of the egg, includes the fertilizing sperm (arrow). The sperm (shown at higher magnification in Fig. 3C) has just entered the egg and is located in the animal hemisphere underneath the egg plasma membrane about 30° from the animal pole. This result, plus the two LM observations of fertilization cones in the animal hemisphere, shows that sperm can fertilize

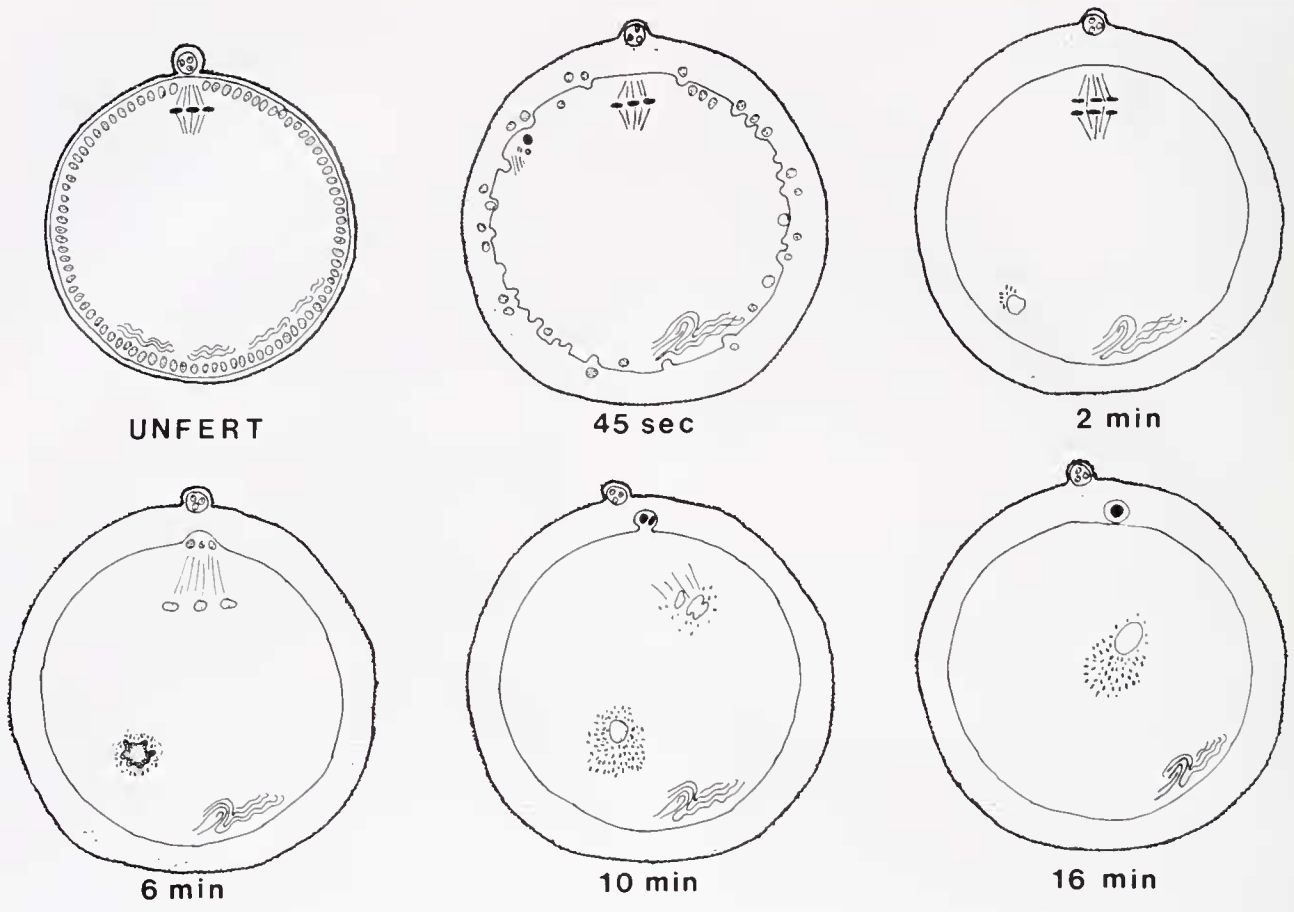


Figure 1. Diagrams of the unfertilized egg and fertilized eggs through 16 min after insemination. The distribution of yolk and the sperm aster are not shown. The unfertilized egg has the first polar body and is in second meiotic metaphase. The egg cortex contains numerous cortical granules, and in the vegetal third of the egg there are sheets of dense granules interleaved with endoplasmic reticulum just beneath the layer of cortical granules. At 45 s, most of the cortical granules have undergone exocytosis, the sperm has entered, the axoneme has largely dispersed, and the sheets of dense granules and endoplasmic reticulum have formed into whorls to constitute the vegetal pole plasm. By 2 min, the egg is in second meiotic anaphase, the cortical reaction is complete, and the sperm nucleus has migrated to the vegetal cytoplasm and formed a small aster, with which a small cluster of mitochondria is associated. By 6 min, the egg is in telophase of the second meiotic division, the sperm nucleus has swollen, and the peripheral chromatin has condensed more than the central chromatin. A cloud of mitochondria surrounds the sperm nucleus. By 10 min, the second polar body has formed. The nuclear envelopes have formed around individual or groups of maternal chromosomes. These chromosome-containing vesicles are fusing to form the maternal pronucleus. The enlarged male pronucleus is surrounded by a larger cloud of mitochondria and has migrated partway towards the female pronucleus. By 16 min, the second polar body has separated from the egg, and the pronuclei have met and are associated with an asymmetric cloud of mitochondria brought by the male pronucleus.

eggs of *Branchiostoma floridae* in the animal hemisphere, although the sample size is far too small to rule out the possibility that sperm can also enter in the vegetal hemisphere.

By 45 s after insemination, the cortical reaction is nearly complete, and only a few unreacted cortical granules remain. The yolk granules are still evenly distributed except in a broad area around the newly entered sperm where they are somewhat sparser (Fig. 3B). At higher magnification, the sperm mitochondrion, one of the two centrioles (the other is out of the plane of section), and microtubules of the axoneme are visible in the egg cytoplasm (Fig. 3C). The nuclear envelope has already disappeared, and the chromatin has decondensed at the nuclear periphery and in patches deeper in the nucleus.

The first polar body adheres to the fertilization envelope as it rises from the egg surface (Fig. 3D). Within the egg, the meiotic spindle, with chromosomes still aligned on the metaphase plate, remains associated with relatively lucent mitochondria and is closely surrounded by yolk

granules (Figs. 3D, 4A). Deeper in the cytoplasm, especially in the animal hemisphere, the mitochondria are frequently aggregated into clusters (Fig. 4D). This arrangement of mitochondria persists at least until first cleavage; there is no apparent movement of mitochondria from the cortical cytoplasm to the vegetal hemisphere to surround the sperm nucleus as occurs in ascidians during ooplasmic segregation. These aggregates of mitochondria and others described below associated with the pole plasm and male pronucleus are very small compared to those in the ascidian myoplasm and are not large enough to be detected in living eggs with fluorescent mitochondrial dyes. Thus both $DioC_3(3)$ and rhodamine 123 seemed to show a uniform distribution of mitochondria for at least 20 min after insemination. Although the subsequent development of eggs in $DioC_3(3)$ was not tested, eggs reared in the dark in rhodamine 123 developed into normal 3-day larvae.

The subcortical sheets of dense granules and associated ER in the vegetal third of the unfertilized egg have come together in a yolk-poor zone of cytoplasm to one side of the vegetal pole; 6 to 10 layers, each composed of a sheet of granules and a cisterna of SER, are roughly spiraled together so that in cross section the pattern resembles that of a fingerprint (Fig. 4C; for higher magnification see appearance at 6 min Fig. 7D). At the periphery of these whorls are numerous mitochondria (Fig. 4C). This reorganization does not appear to be associated with a massive inflow of materials from other regions of the egg. Because of its location, we will call this specialized region of cytoplasm the vegetal pole plasm. From this point in development, the appearance of these whorls remains relatively constant, at least through formation of the zygote nucleus. The pole plasm is visible in toluidine blue-stained, 2 μm sections as reddish-purple strands in a yolk-free zone near the vegetal pole. At each cleavage, at least through the early blastula, it is segregated into a single blastomere (data not shown).

Sperm pronucleus near vegetal pole: 2–6 min after insemination (Figs. 1, 5)

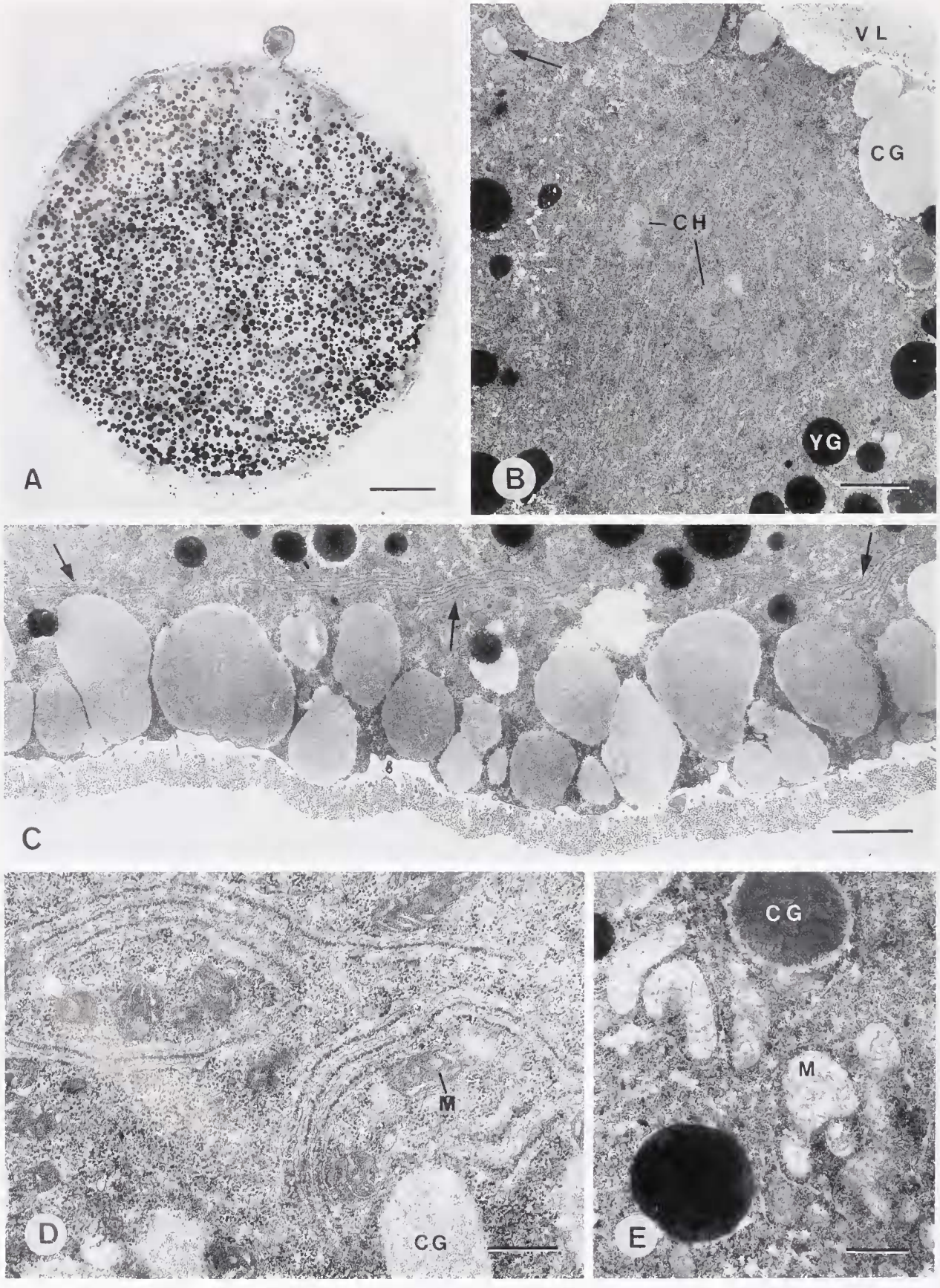
By 2 min after insemination, the male pronucleus, regardless of the point of sperm entry, is located in the egg cortex near the vegetal pole. In TEM sections of an egg at 2 and one at 6 min after insemination (Figs. 5A, 6A), and in LM sections through ten eggs at 3 to 6 min after insemination, the male pronucleus was always near the vegetal pole. These results are consistent with previous LM studies demonstrating that the swollen male pronucleus first becomes visible near the vegetal pole of the egg of *Branchiostoma lanceolatum* (van der Stricht, 1985; Sobotta, 1897; Cerfontaine, 1906). Presumably, as in ascidian eggs (Speksnijder *et al.*, 1989), sperm entering the animal hemisphere of the ascidian egg are rapidly translocated close to the vegetal pole. At 2 min after insemination, the male pronucleus is about 2.5 μm in diameter and is less dense than the cytoplasm (Figs. 5A, B). There is no trace of a nuclear envelope surrounding the male chromatin (Fig. 5B, insert). A sperm aster is present near the male pronucleus (Fig. 5D) (none of our sections went through both the male pronucleus and the aster). We did not see the male centrioles at this time; presumably the sections containing them were lost. The cytoplasm around the male pronucleus is not enriched in mitochondria, as it is in ascidians, although a few mitochondria are aggregated at the convergence of the astral rays (Fig. 5D).

In the cytoplasm, yolk granules are somewhat less numerous in the animal hemisphere than in the vegetal hemisphere (Fig. 5A). This is the opposite of the situation in ascidians where the yolk is concentrated in the animal hemisphere during the first phase of ooplasmic segregation. The meiotic spindle is still present at the animal pole, but the female chromosomes, which are inconspicuous, have begun to migrate to opposite poles of the spindle (data not shown). The pole plasm with its sheets of dense granular material is unchanged from 45 s after insemination (see appearance at 6 min, Figs. 7A, B).

Beginning of pronuclear migration: 6–10 min after insemination (Figs. 1, 6, 7)

By 6 min after insemination, the male pronucleus, which has enlarged to 5 μm in diameter and developed an irregularly lobed outline, has migrated from close to the egg cortex about 30 μm towards the center of the egg (Figs. 1, 6A). The chromatin of the male pronucleus remains decondensed at the center, but has become more condensed in the peripheral lobes and is partly bounded by a nuclear envelope (Fig. 7F, arrow). Numerous mitochondria, apparently recruited from the cytoplasm in the vegetal hemisphere, closely surround the periphery of the male pronucleus (Fig. 7B). Although the sperm aster was not seen at this stage, as in the preceding stage (see Fig. 5D) it is likely that the aggregate of mitochondria also converges upon the sperm aster.

The maternal chromosomes have moved apart on the meiotic spindle. Those destined to form the female pronucleus are rounded, about 6 μm in diameter, and lie in a yolk-poor zone slightly away from, and to one side of, the animal pole (Figs. 6A, C; 7A). A few fragments of nuclear envelope have formed at the periphery of the chromosomes, which are still associated with spindle fibers (Fig. 7A). At the animal pole, there is a bulge in the egg surface, evidently the beginning of the second polar body (Fig. 6D). From previous observations, this polar body forms about 8 min after insemination (Holland and Hol-


A
B
VL
CG
CH
YG
C
D
M
CG
E
CG
M

land, 1989a). As in the preceding stage, yolk granules are somewhat scarcer in the animal hemisphere (Fig. 6A), and the pole plasm is prominent near the vegetal pole (Figs. 6B; 7C, D).

Formation of the female pronucleus and migration of pronuclei: 10–16 min after insemination (Figs. 1, 8, 9)

By 10 min after fertilization, the male pronucleus has continued its migration towards the female pronucleus and enlarged to about 6 μm in diameter. (Fig. 8B, C). The chromatin is of uniform density, similar to that of the peripheral lobes at 6 min after insemination, and the nucleus is bounded by a nuclear envelope (Fig. 8C). The area of yolk-free cytoplasm around the male pronucleus has enlarged to about 15 μm in diameter and has become rich in mitochondria; there are about four mitochondria per μm^2—approximately ten times the concentration elsewhere in the cytoplasm.

The second polar body has formed, but is still attached to the egg (Figs. 8A, 9D). At the point of attachment, there is a prominent density (Zwischenkörper) through which the spindle microtubules pass (Fig. 9D, insert). In the polar body, there are several chromosome-containing vesicles, each bounded by its own nuclear envelope (Fig. 9D).

Within the egg, individual female chromosomes or groups of chromosomes have become surrounded by nuclear envelopes. Depending on the egg, these chromosome-containing vesicles are either in the process of fusing and are still located close to the animal pole (Fig. 9A–C) or they have completed fusing into a single female pronucleus 5.5 × 8 μm in diameter, which has migrated somewhat off-center to just above the equator (Figs. 1, 8B, D). The female pronucleus does not migrate further. The nuclear matrix is of low electron density but contains small scattered areas of higher density (Figs. 8D; 9B, C). The female pronucleus is in a large, irregular area of yolk-poor cytoplasm, which extends to the yolk-poor cytoplasm near the animal pole (Fig. 8B). Although mitochondria are not uncommon near the female pronucleus, they are four to five times less numerous than those around the male pronucleus (compare Fig. 8D and C).

The pole plasm (Figs. 1; 8A; 9E, F) is little changed from earlier times. However, in some places, the strands of dense material are no longer closely associated with ER and have lost their parallel relation to one another (Fig. 9E, F).

Pronuclear fusion: 16 min after insemination (Figs. 1, 10)

By 16 min after insemination, the male pronucleus has migrated to, and fused with, the female pronucleus (Figs. 1; 10A, E, F). The resulting zygote nucleus, which is about 8 × 12 μm in diameter, lies in a zone relatively free of yolk about 17 μm in diameter in the animal hemisphere just above the equator, about half-way between the edge and the center of the egg. At one side of the nucleus, presumably that deriving from the male pronucleus, is a large aggregate of mitochondria (Fig. 10E). Thus a mitochondria-rich zone of cytoplasm surrounds the newly formed zygote nucleus in both lancelets and ascidians. However, this zone is vastly larger in ascidians, and comes not from the vegetal cytoplasm as the male pronucleus migrates through it, but from the cortical cytoplasm, which collects around the male pronucleus during ooplasmic segregation (Zalokar and Sardet, 1984).

The zygote nucleus contains a few small, dense inclusions like those previously described in the female pronucleus (Fig. 10F) and is bounded everywhere by a nuclear envelope (Fig. 10E, F). Although some microtubules, evidently part of the astral rays, were seen near the nucleus, no centrioles were encountered in our sections.

By 16 min, the second polar body has separated from the egg, but their plasma membranes remain closely apposed (Fig. 10B–D). The second polar body tends to remain at the animal pole during the cleavage stages, although Hirakow and Kajita (1991) sometimes observed it in other locations. Within the polar body, the chromosome-containing vesicles have fused into a single nucleus.

Discussion

Previous work on the early embryology of lancelets has been largely on the European species, *Branchiostoma lanceolatum* (Wilson, 1893; van der Stricht, 1896; Sobotta, 1897; Cerfontaine, 1906; Conklin, 1932, 1933), and to a

Figure 2. TEMs of unfertilized eggs with animal pole uppermost. A. Central section through the first polar body at top adjacent a small yolk-free area including the second meiotic spindle. At this magnification, the spindle fibers and chromosomes cannot be resolved. Scale bar: 20 μm. B. Higher magnification of the second meiotic spindle. The first polar body is not in the plane of section. Mitochondria near the meiotic spindle generally have an electron lucent matrix (arrow). Scale bar: 2 μm. C. The vegetal pole. Parallel sheets of dense granules interleaved with endoplasmic reticulum (arrows) lie just beneath the layer of cortical granules. Scale bar: 2 μm. D. Higher magnification of the sheets of dense granules and associated endoplasmic reticulum at the vegetal pole. Mitochondria (M) have a relatively electron-dense matrix. Scale bar: 0.5 μm. E. Higher magnification of the electron lucent mitochondria (M) near the animal pole. Scale bar: 0.5 μm. Chromosomes (CH), cortical granule (CG), vitelline layer (VL), yolk granule (YG).

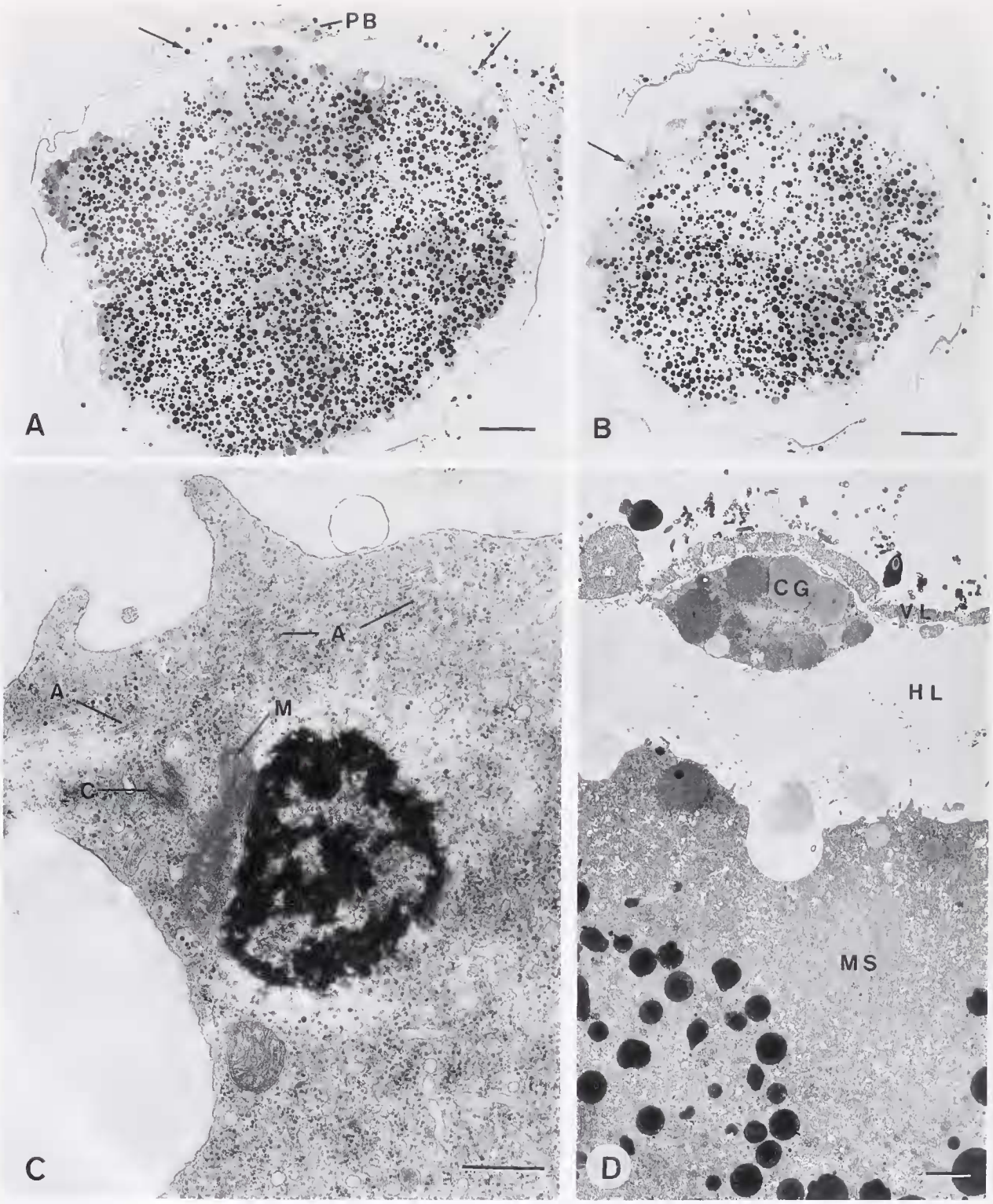


Figure 3. TEMs of eggs at 45 s after insemination. A. Section through the first polar body (PB) at top. The cortical reaction is in progress. Many supernumerary sperm (arrows) are associated with the rising fertilization envelope. The plane of section does not pass through the meiotic spindle. Scale bar: 20 μm. B. Section through the same egg as in (A) in the same orientation about half way between the center of the egg and the periphery. The fertilizing sperm (arrow) has entered into the animal hemisphere, and the yolk is patchy in its neighborhood. Scale bar: 20 μm. C. Higher magnification of the fertilizing sperm in (B). The nucleus is associated with the sperm mitochondrion (M) and centriole (C). The nuclear envelope has disappeared and the chromatin has begun to decondense. The axoneme (A) has largely dispersed. Scale bar: 0.5 μm. D. The first polar body, which contains several unreacted cortical granules (CG), and whorls of nuclear envelope (arrow) is sandwiched between the vitelline layer (VL) and material derived from the cortical granules, the hyaline layer (HL). Yolk granules closely surround the meiotic spindle (MS). Scale bar: 2.0 μm.

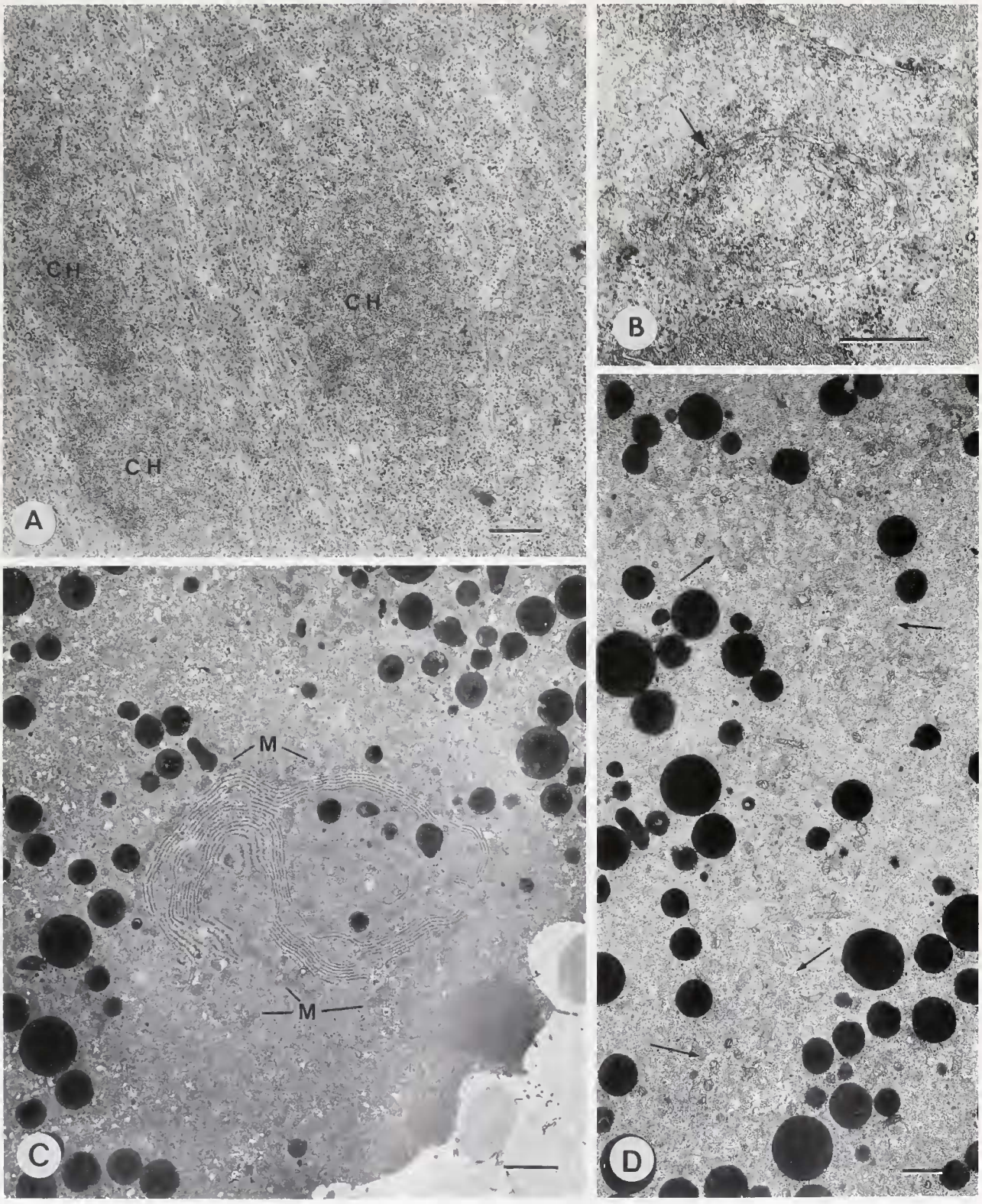


Figure 4. TEMs of eggs at 45 s after insemination. A. Higher magnification of chromosomes (CH) on the meiotic spindle. Scale bar: 0.5 μm. B. Higher magnification of the polar body in 3D showing the whorl of nuclear envelope (arrow). Scale bar: 0.5 μm. C. The sheets of dense granules, endoplasmic reticulum, and mitochondria (M) that constitute the vegetal pole plasm. Scale bar: 2 μm. D. Patchy yolk distribution and aggregated mitochondria (arrows) in the animal hemisphere. Scale bar: 1 μm.

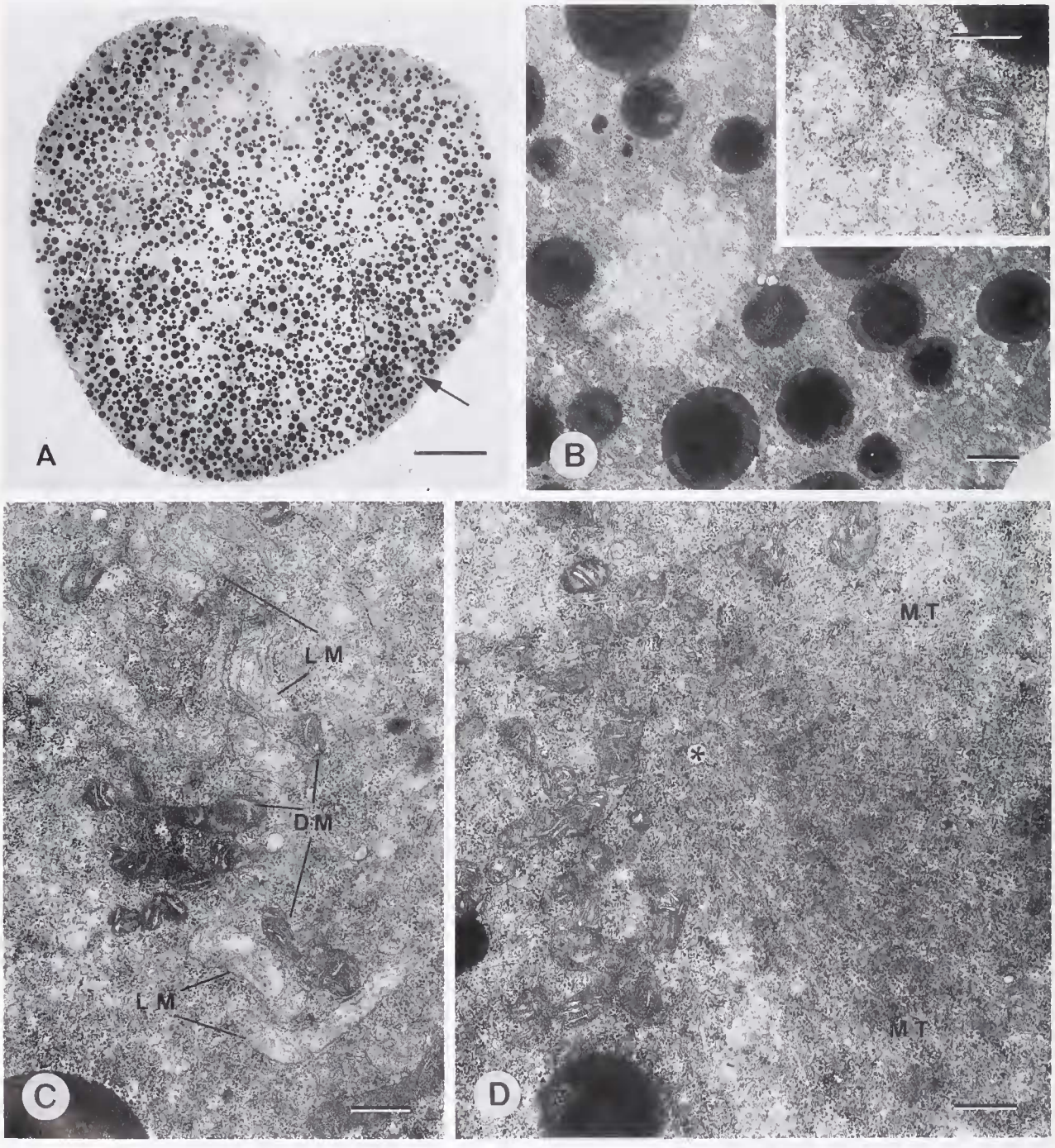


Figure 5. TEMs of eggs at 2 min after insemination. A. Cross section through meiotic spindle (top) and the nucleus of the fertilizing sperm (arrow). The cortical reaction is complete. The indentation in the egg at the animal pole is probably an artifact due to the hypertonicity of the fixative. The polar body and fertilization envelope are not in the figure. Scale bar: 20 μm. B. Higher magnification of the fertilizing sperm nucleus in (A). The aster is out of the plane of section. Scale bar: 1 μm. (B, inset) The edge of the male nucleus at higher magnification. There is no nuclear envelope. Scale bar: 0.5 μm. C. Co-occurrence of mitochondria with dense matrix (DM) and lucent matrix (LM) near the animal pole. Scale bar: 0.5 μm. D. The aster associated with the male nucleus in (D). There is a small cluster of mitochondria where the astral microtubules (MT) converge (asterisk). The male nucleus and centrioles are out of the plane of section. Scale bar: 0.5 μm.

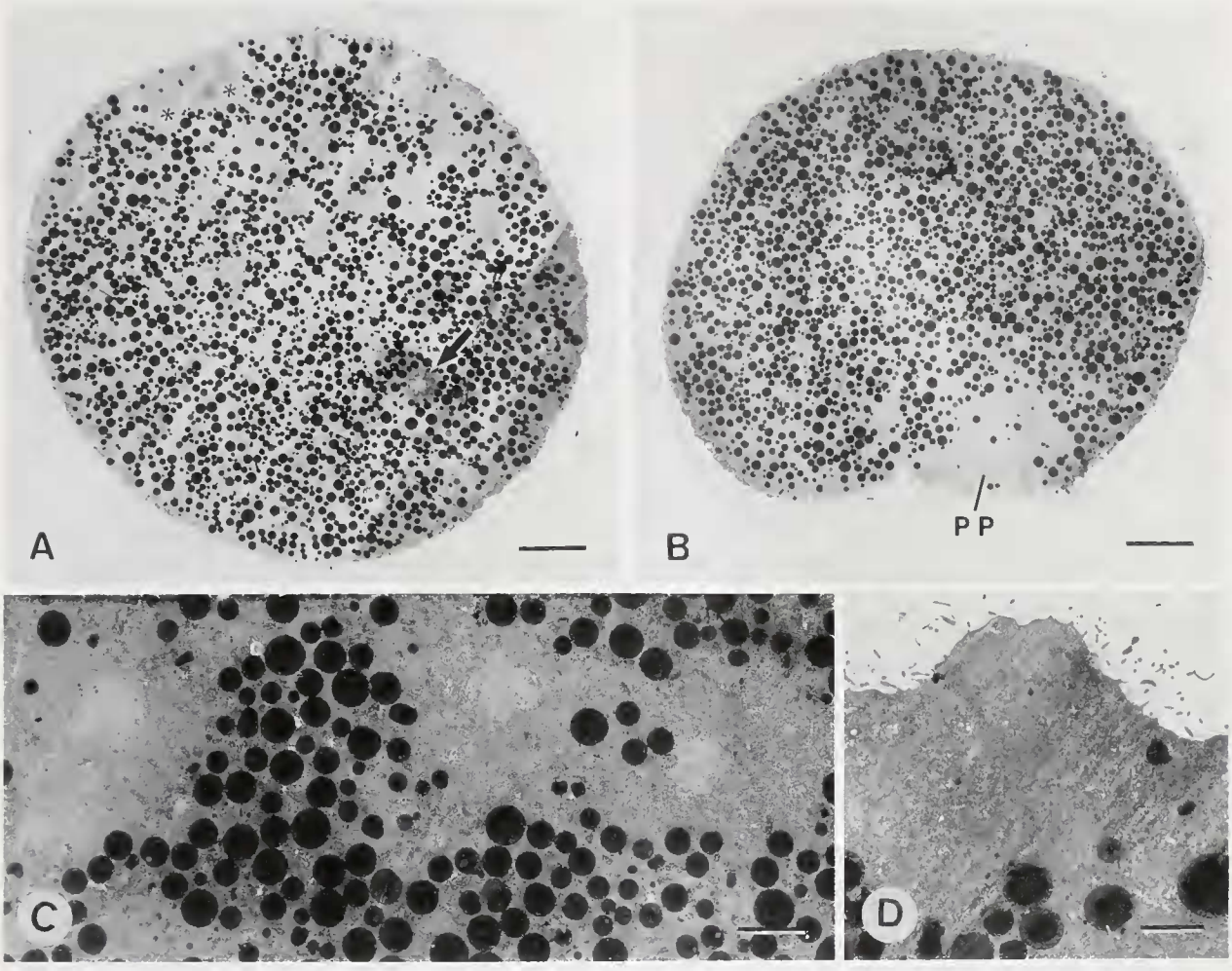


Figure 6. TEMs of eggs fixed at 6 min after insemination. A. Section through the male pronucleus (arrow). Animal hemisphere is uppermost. The section does not pass through the meiotic spindle and polar body. The female chromosomes are located about 11 o'clock near the animal pole (asterisks), but are not visible at this magnification due to their low contrast. Scale bar: 20 μm. B. Section through the same egg as in (A) that passes through the vegetal pole plasm (PP). The indentation at the vegetal pole is probably an artifact due to the high tonicity of the fixative, but marks the site of the future cleavage furrow. Scale bar: 20 μm. C. Three female chromosomes from the same egg as in A and B. Scale bar: 4 μm. D. Bulge at the animal pole at the site of formation of the second polar body. The polar body chromosomes are not in the plane of section. Scale bar: 2 μm.

lesser extent, on the Asian species, *B. belcheri* (Tung *et al.*, 1958, 1960a, b, 1962a, b; Hirakow and Kajita, 1990, 1991). There are no marked differences between these species. Thus, although aside from a few micrographs of Hirakow and Kajita (1990), our work on *B. floridae* is the only TEM study on the earliest embryonic stages, and it is likely that our results also apply to other species of *Branchiostoma;* the largest ovarian oocytes have virtually the same fine structure in *B. floridae, B. lanceolatum,* and *B. belcheri* (reviewed in Holland and Holland, 1991), as do the blastomeres of *B. floridae* and *B. belcheri* (Hirakow and Kajita 1990, 1991; Holland and Holland, unpub.).

Position of sperm entry; formation and migration of the pronuclei

Sobotta (1897) depicted a sperm entering a lancelet egg with its tail extending into the perivitelline space and its nucleus with the same size and staining properties as a yolk granule. He maintained that, although sperm can enter the egg anywhere on the surface, they usually do so near the vegetal pole. He, along with van der Stricht (1896) and Cerfontaine (1906), thought that the fertilizing sperm first developed into a dark-staining irregular mass near the vegetal pole before swelling into a clear, spherical pronucleus. This observation led van der Stricht (1896), Cer-

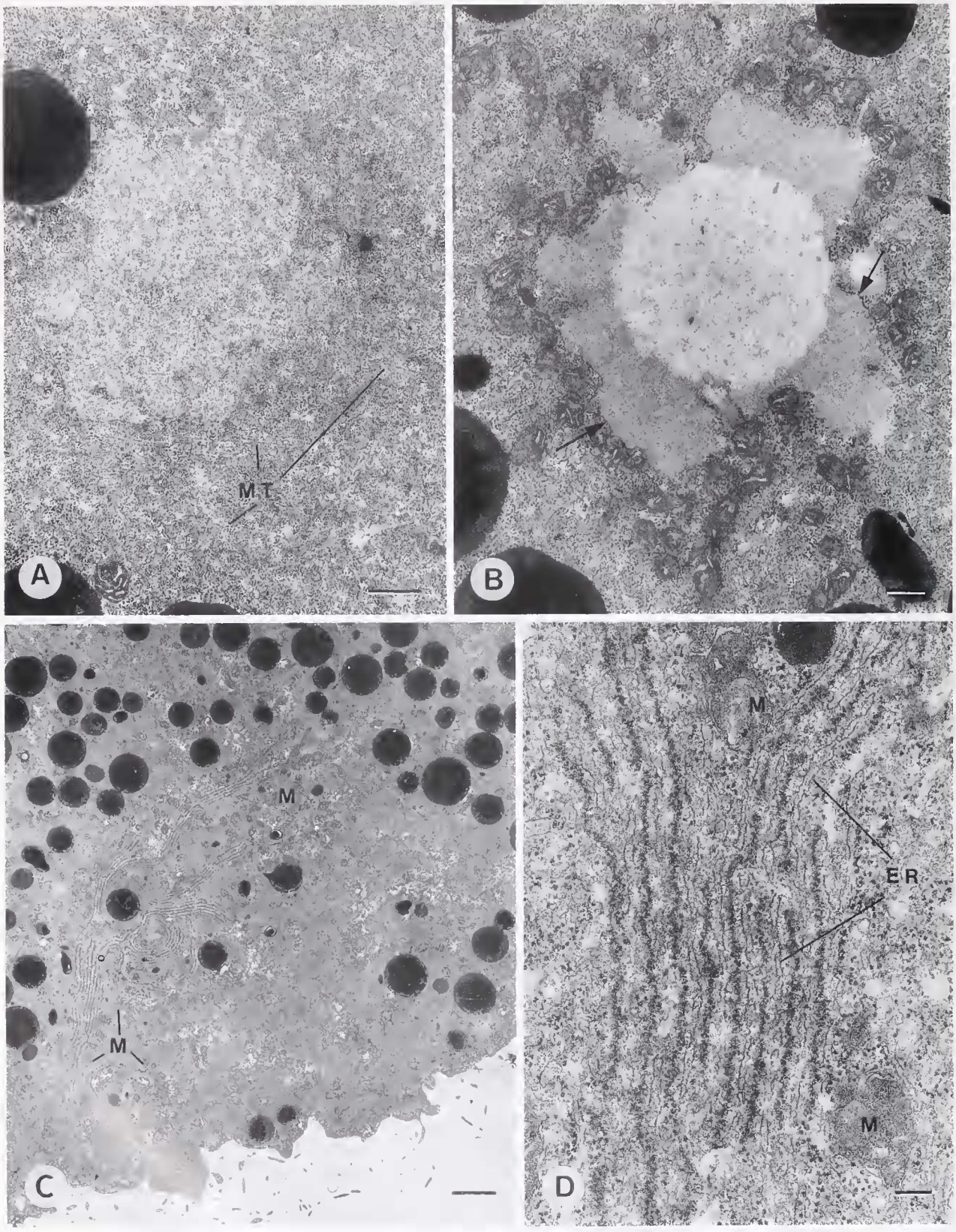


Figure 7. TEMs of eggs at 6 min after insemination. A. Higher magnification of the female chromosome at the far right in Figure 6C. There is no nuclear envelope. The chromosome is still associated with microtubules of the meiotic spindle (MT). Scale bar: 1 μm. B. Higher magnification of the male pronucleus in Figure 6A. It is closely surrounded by mitochondria, and a partial nuclear envelope has formed (arrows). The aster is not in the plane of section. Scale bar: 0.5 μm. C. Higher magnification of the vegetal pole plasm in Figure 6B. Numerous mitochondria (M) are associated with the sheets of dense granules. Scale bar: 2 μm. D. High magnification of the vegetal pole plasm in C. Mitochondria (M) are closely associated with sheets of endoplasmic reticulum (ER) that lie in between the sheets of dense granules. Scale bar: 0.2 μm.

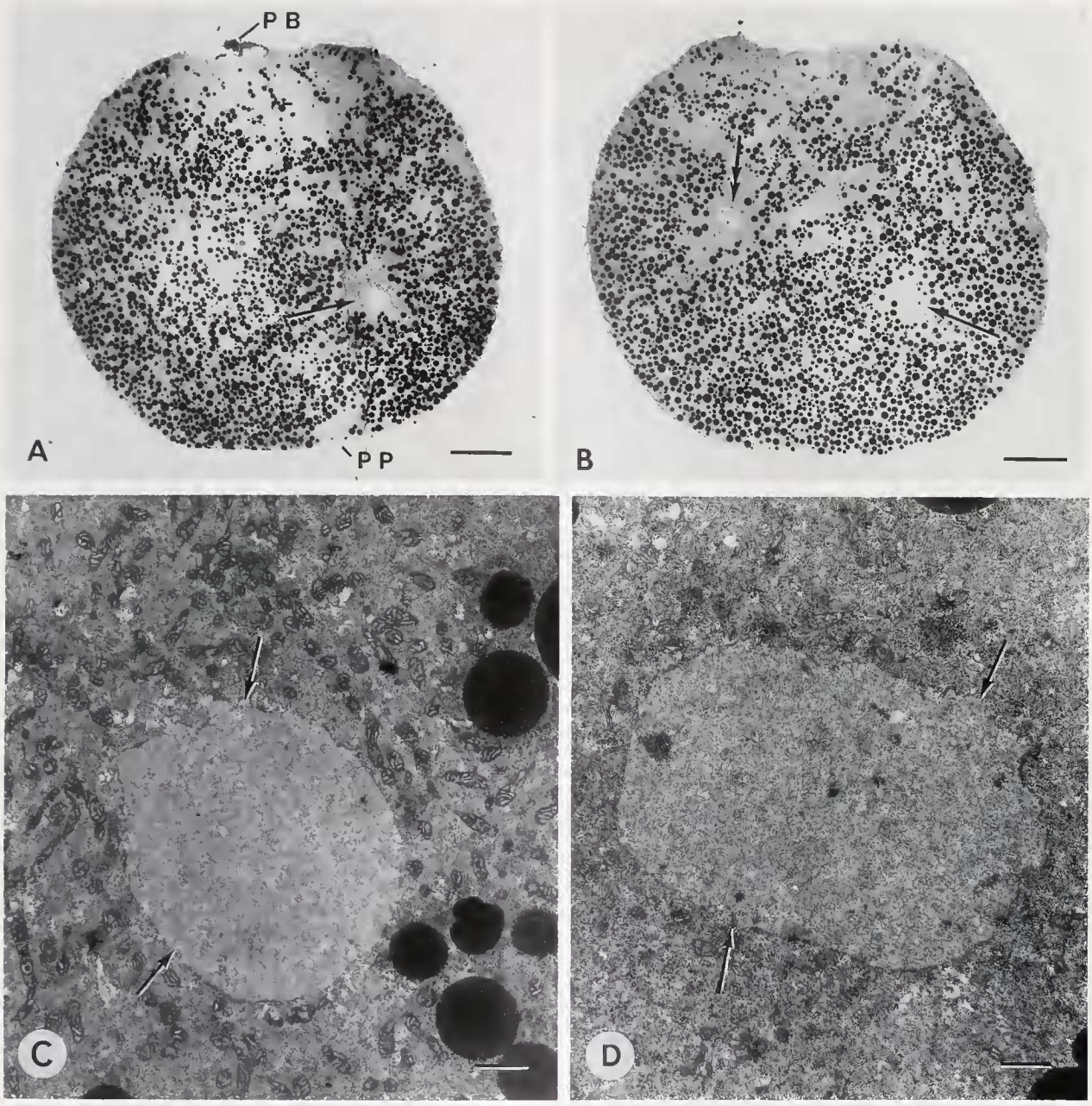


Figure 8. TEMs of eggs at 10 min after insemination. A. Section through the second polar body (PB), male pronucleus (arrow), and vegetal pole plasm (PP). Black dots in the yolk-free zone around the male pronucleus are mitochondria. The yolk is patchily distributed in the animal hemisphere. Scale bar: 20 μm. B. Section through the same egg as in A, about 10 μm deeper, which passes through the female pronucleus (double arrow). The yolk-free patch of cytoplasm underlying the male pronucleus is at lower right (single arrow). Scale bar: 20 μm. C. Higher magnification of the male pronucleus and its cloud of mitochondria in A. The nuclear envelope is complete except in a few spots (arrows). Scale bar: 1 μm. D. Higher magnification of the female pronucleus in B. The nuclear envelope is complete except in a few areas (arrows). The nucleus contains a few dense patches. Scale bar: 1 μm.

fontaine (1906), and Conklin (1932) to conclude that the sperm *always* enters near the vegetal pole. In contrast, our results show that the fertilizing sperm can enter the egg near the animal pole (three out of three observations), although more extensive study would be required to show whether they always do so. The axoneme enters with the nucleus, mitochondrion, and centrioles, but rapidly disappears. Then the sperm nucleus undergoes two phases

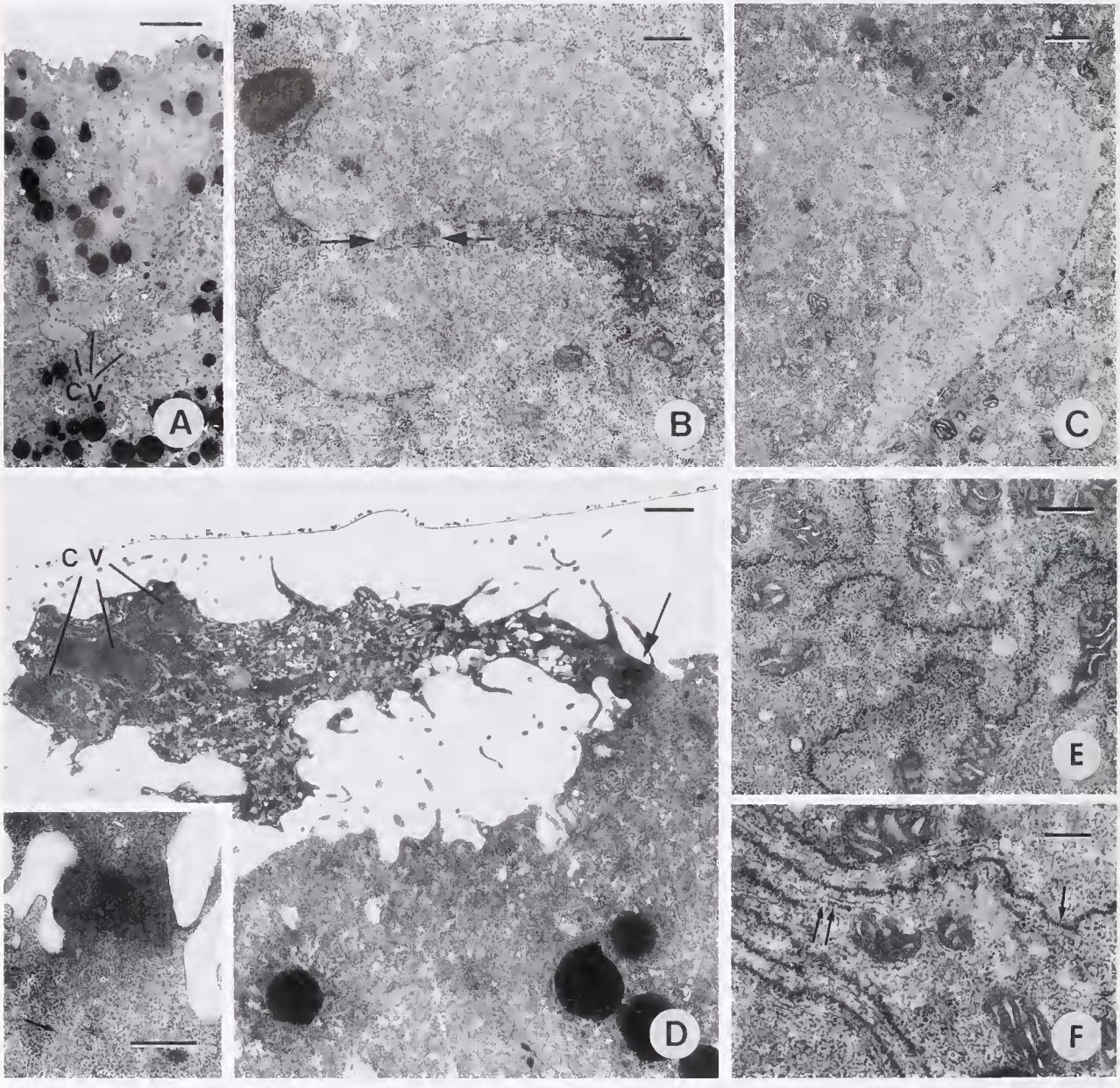


Figure 9. TEMs of eggs at 10 min after insemination. A. Section near the animal pole with three maternal chromosome-containing vesicles (CV). Scale bar: 4.5 μm. B. Higher magnification of two of the maternal chromosome-containing vesicles in A, which have begun to fuse at arrows. Scale bar: 0.5 μm. C. Two maternal chromosome-containing vesicles that have fused and are connected by a broad bridge. Scale bar: 0.5 μm. D. Higher magnification of the second polar body in Figure 8A. Three chromosome-containing vesicles (CV) are visible within it. Where it is connected to the egg there is a dense Zwischenkörper (arrow). Scale bar: 1 μm. (Insert) The Zwischenkörper at higher magnification showing the microtubules (arrow) remaining from the meiotic spindle. Scale bar: 0.5 μm. E. Sheets of dense granules and associated mitochondria in the vegetal pole plasm that are no longer associated with endoplasmic reticulum. Scale bar: 0.5 μm. F. Sheets of dense granules and mitochondria in the vegetal pole plasm in relatively close association with smooth endoplasmic reticulum in some places (twin arrow), but not in others (single arrow). Scale bar: 0.3 μm.

of migration. First, between 45 s and 2 min after insemination, the male pronucleus evidently migrates rapidly to the vicinity of the vegetal pole. Second, between 6 min and 16 min, the male pronucleus migrates slowly back into the animal hemisphere to meet the female pronucleus.

Soon after entering the egg, the sperm nucleus rapidly decondenses, staining less intensely with toluidine blue,

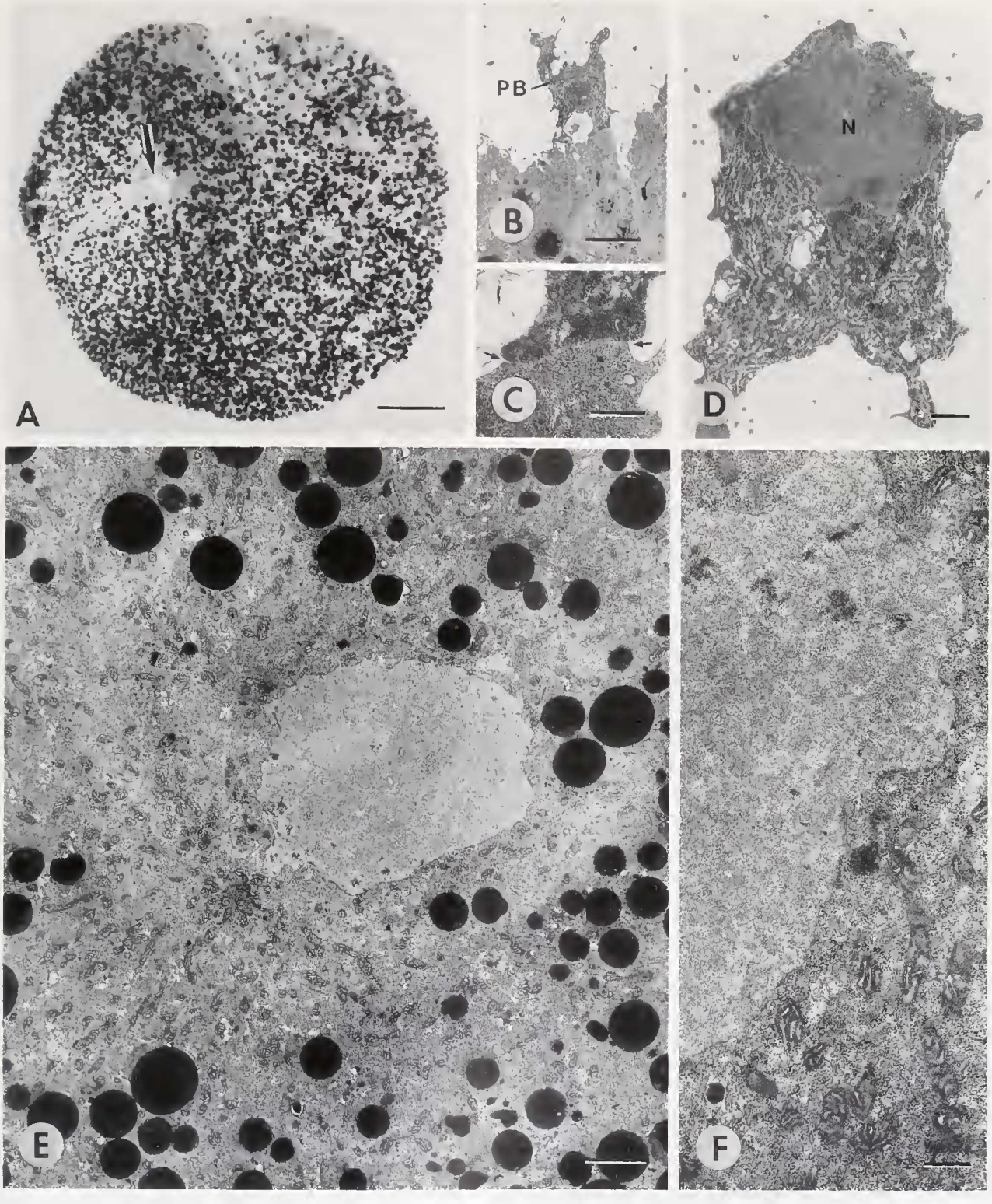


Figure 10. LM (A) and TEMs (B–F) of eggs at 16 min after insemination. A. Section through the animal pole (top) and zygote nucleus (arrow). The yolk remains patchy near the animal pole. Scale bar: 20 μm. B. The proximal part of the second polar body (PB), which has detached from the egg. Scale bar: 5 μm. C. The polar body and egg, although no longer in cytoplasmic continuity, remain very tightly apposed (arrows). Scale bar: 1 μm. D. The distal part of the second polar body. Some of the chromosome-containing vesicles have fused into a nucleus (N). Scale bar: 1 μm. E. The portion of the zygote nucleus probably derived from the male pronucleus has a cloud of mitochondria at one side. Scale bar: 12 μm. F. A portion of the zygote nucleus probably derived from the female pronucleus. There are dense patches within the nucleus and the nuclear envelope is continuous. Scale bar: 0.5 μm.

and cannot be seen by LM until 4–5 min after insemination, when it has swollen considerably. No part of the fertilizing sperm ever becomes the large, dark-staining irregular structure reported by earlier embryologists—this structure is clearly not the sperm at all, but the vegetal pole plasm. The sheets of dense granules belonging to the vegetal pole plasm are certainly responsible for the mistaken view of van der Stricht (1896) and Cerfontaine (1906) that the sperm tail enters along with the head and remains behind near the vegetal pole as a sperm remnant after the male pronucleus swells, develops an aster, and begins its slow migration. Apparently, both Sobotta (1897) and Conklin (1932) overlooked the vegetal pole plasm in eggs with large pronuclei and thus were spared the difficulty of having to explain its presence. The female pronucleus and the swollen male pronucleus are readily visible by LM, and their migrations were correctly described by Sobotta (1895, 1897), van der Stricht (1896), and Cerfontaine (1906).

The second phase of male pronuclear migration begins just before the second polar body forms. The female chromosomes then move to one side of the animal-vegetal axis and join to form a female pronucleus, which migrates to just above the equator to be met by the male pronucleus. Cerfontaine (1906) and Conklin (1932) believed that, as in ascidians, the site where the pronuclei meet is the posterior region of the future embryo. However, in the absence of obvious cytoplasmic markers of either the posterior or anterior poles, it is puzzling how they could make the distinction except by analogy with ascidians, some species of which have yellow pigment granules localized in the egg before cleavage at the posterior pole of eggs and embryos (Conklin, 1905a). Conklin (1932) thought he could distinguish a similar, although less conspicuous, marker of the posterior pole in lancelets; however, as discussed below, the existence of such a marker is most unlikely.

Pronuclear migrations in lancelets and ascidians are similar, although some of the details vary. In ascidians (Conklin, 1905a), as in lancelets, it was formerly believed that the sperm enters near the vegetal pole. However, it has since been shown that ascidian sperm can fuse with all regions of the egg plasma membrane (Ortolani, 1958; Talevi and Dale, 1986), but preferentially enter the animal hemisphere (Speksnijder *et al.,* 1989). In ascidians, as in lancelets, there are two phases of sperm migration. First, the sperm is rapidly transported close to the vegetal pole. Staining with DNA-specific dyes and an anti-tubulin antibody has shown that during this phase, the ascidian sperm nucleus remains condensed and is accompanied by the axoneme (Sawada and Schatten, 1988)—in contrast, as we have demonstrated, soon after entering the egg, the lancelet sperm nucleus decondenses and the axoneme disappears. In both ascidians and lancelets, the male pronucleus, once in the vegetal cytoplasm, swells, develops a large aster, and then, in a second slower phase of migration, moves towards the animal pole. The pronuclei meet just below the equator in ascidians and just above it in lancelets. An aggregation of mitochondria accompanies the male pronucleus in this migration. However, the mitochondria are far more numerous in ascidians than in lancelets and are derived, not by gradual recruitment from the surrounding cytoplasm, but from the mitochondria-rich cortical cytoplasm, which flows along with the male pronucleus from the animal hemisphere to the vegetal pole and then to the posterior pole to form the myoplasmic crescent (Sawada and Schatten, 1988; Speksnijder *et al.,* 1989).

The mechanism for migration of the pronuclei in lancelets is unclear. In ascidians, the first phase of male pronuclear migration occurs concomitantly with a dramatic shape change and segregation of ooplasm (Jeffery, 1984), all of which are inhibited by cytochalasin and are thus probably mediated by the contraction of cortical microfilaments (Sawada, 1988; Sardet *et al.,* 1989). We did not test whether cytochalasin could prevent the first phase of sperm migration in the lancelet; however, lancelet eggs undergo neither a shape change (Holland and Holland, 1989a) nor obvious ooplasmic segregation.

In ascidians, the sperm aster is necessary for the second phase of migration of the male pronucleus and for the movement of the mitochondria-rich myoplasm from the vegetal pole towards the posterior pole; both movements are prevented by agents that disrupt microtubules (Manes and Barbieri, 1977; Sawada and Schatten, 1988). Whether microfilaments are also involved is not known. However, in sea urchins, migration of the male pronucleus, which also depends on microtubules, is independent of microfilaments (Schatten and Schatten, 1981). Thus, in lancelets as well, although microtubule inhibitors have not been tested, the sperm aster is probably necessary for migration of the male pronucleus; the sperm aster is also probably responsible for the aggregation of mitochondria around the male pronucleus. Mitochondria do not aggregate around the female pronucleus, which lacks an aster, or around the male pronucleus before the aster forms. In addition, in somatic cells, mitochondria are frequently seen in close association with microtubules (Heggeness *et al.,* 1978), which have been shown to function as tracks for the movement of organelles, particles, and molecules in somatic cells, eggs, and embryos (Schliwa, 1984; Vale *et al.,* 1985; Hamaguchi *et al.,* 1986; Kobayakawa, 1988; Ressom and Dixon, 1988; Yisraeli *et al.,* 1989).

Vegetal pole plasm

As mentioned above, the vegetal pole plasm was seen with LM in lancelet eggs but misidentified as the fertilizing

sperm (van der Stricht, 1896; Sobotta, 1897; and Cerfontaine, 1906). With TEM, Hirakow and Kajita (1990) illustrated the pole plasm in fertilized, uncleaved eggs in their figure 12, but interpreted it as an "unusual stack of rough endoplasmic reticulum rarely encountered."

The vegetal pole plasm of lancelets has the components typical of pole plasms in other organisms, *i.e.,* numerous mitochondria, conspicuous aggregates of dense fibrogranular material, and profiles of endoplasmic reticulum. The precise configuration of the pole plasm in lancelets, however, has not been described in any other organism. Among the deuterostomes, vegetal pole plasm has been seen only in chaetognaths and anuran amphibians. It does not occur in appendicularian (Holland *et al.,* 1988) or ascidian tunicates, or in echinoderms; its possible presence in hemichordates has not been investigated (Eddy, 1975). In many organisms, the vegetal pole plasm is destined to be included in the primordial germ cells and, thus, is termed "germ plasm." The germ plasm is enriched in RNA, and some mRNAs and proteins specific to it have been identified (Phillips, 1982, 1985; Yamaguchi *et al.,* 1983; Hay *et al.,* 1988; Nakazato and Ikenishi, 1989). Nevertheless, it is not known how the germ plasm acts in germ cell determination for any animal (Davidson, 1986).

The germ cells are typically endodermal derivatives in animals with germ plasm, *e.g.,* chaetognaths and anurans, but are usually mesodermal derivatives in those lacking germ plasm, *e.g.,* urodele amphibians and probably ascidians (Nieuwkoop and Sutasurya, 1976, 1979; Nieuwkoop, 1991). For the lancelet *Branchiostoma belcheri* at the 32 cell-stage, the most vegetal tier of blastomeres, one of which presumably contains the vegetal pole plasm, is destined to form endodermal structures such as the gut; embryos lacking these blastomeres rarely form endodermal structures (Tung *et al.,* 1960a). Thus, the vegetal pole plasm of lancelets may be included in endodermal cells, and the germ cells would thus be expected to be endodermal in origin. In *B. lanceolatum,* Boveri (1892) found primordial germ cells in segmentally arranged outpocketings of the myocoel in relatively late larvae, which suggested to Nieuwkoop and Sutasurya (1979) that the germ cells would be mesodermal, not endodermal, derivatives. However, Boveri lacked earlier larvae and thus could not have determined if the germ cells had arisen in the myocoel or migrated from elsewhere. The possibility that the vegetal pole plasm in lancelets is incorporated into the germ cells merits investigation. The first two blastomeres, when separated, can each give rise to a normal larva (Wilson, 1893; Conklin, 1933; Tung *et al.,* 1958); however, no such embryo has been raised long enough to determine if gonads formed.

The dense granular material in vegetal pole plasm or germ plasm is thought to be related to, and possibly derived from, nuage—dense fibrogranular aggregates containing protein or RNA that frequently occur in association with mitochondria near the nucleus of growing oocytes. Nuage occurs in lancelets (Guraya, 1983; Aizenstadt and Gabaeva, 1987; Holland and Holland, 1991) and in most other organisms that have germ plasm. Nuage is also present in many animals lacking germ plasm, including echinoderms, and among the chordates, ascidians, reptiles, and birds (Eddy, 1975). In the European lancelet *Branchiostoma lanceolatum,* Guraya (1968, 1979) found that nuage contained protein, lipoprotein, and RNA. In mid-oogenesis, the aggregates of nuage break up and are distributed throughout the cytoplasm, becoming localized in the cytoplasm at the vegetal pole in the largest oocytes (Guraya, 1983). At least part of the nuage may be the source of the sheets of dense aggregates present just interior to the cortical granules in *B. floridae,* which coalesce after insemination to form the vegetal pole plasm.

Cytoplasmic specializations at the animal pole

Eggs of *Branchiostoma floridae* have two specializations near the animal pole: first, in both unfertilized and fertilized eggs, animal pole mitochondria are relatively electron-lucent, and second, in fertilized eggs, the yolk becomes patchy in the animal hemisphere. In blastulae of axolotls, there is a similar animal-vegetal difference in mitochondria; those in animal cells are larger and have a much less dense matrix than those of vegetal pole cells (Nelson *et al.,* 1982).

Relatively yolk-poor areas at the animal pole (namely, animal pole plasms) have been described in fertilized eggs of both invertebrates and vertebrates, for example, oligochaetes (Shimizu, 1989), lampreys (Nicander *et al.,* 1968), and amphibians (Wakahara, 1989). Typically, these areas appear either during the meiotic divisions or soon after fertilization. In ascidians, after germinal vesicle breakdown, the material from the germinal vesicle becomes localized at the animal pole. Following fertilization, this yolk-poor cytoplasm follows the myoplasm to the vegetal hemisphere and then migrates with the male pronucleus back towards the animal hemisphere, finally coming to surround the zygote nucleus (Conklin, 1905a, b; Jeffery, 1984). In contrast, in lancelets, the yolk-free patches at the animal hemisphere do not appear to derive from the germinal vesicle. Cerfontaine (1906) mistakenly depicted the remnant of the germinal vesicle persisting to one side of the meiotic spindle; when the germinal vesicle breaks down, however, its substance rapidly blends with the cytoplasm, and the yolk becomes uniformly distributed except immediately around the second meiotic spindle (Conklin, 1933; the present work).

The yolk-free patches that develop after fertilization in the animal hemisphere near the newly entered sperm do not follow it to the vegetal pole. Instead, a yolk-free area

forms *de novo* around the male pronucleus as the second phase of migration begins. The yolk-free patches near the animal pole may form either by one or more of the following: (1) an expansion of the egg volume at the animal pole, or (2) an aggregation and movement of yolk towards the vegetal pole, or (3) a movement of yolk-free cytoplasm to the animal pole. The last explanation is perhaps the most likely because in many eggs (*e.g.*, barnacles, oligochaetes, lampreys, and teleost fish) there is such a flow of cytoplasm to the animal pole from the interior or from the peripheral layers of the egg (reviewed by Wall, 1990).

The animal cytoplasm has been studied much less than the vegetal cytoplasm in regard to its role in embryogenesis. In general, the animal cytoplasm is destined to form ectoderm. In lancelets, the most animal of the four tiers of blastomeres at the 32-cell stage, if isolated, forms only epidermal structures; however, removal of this tier does not affect the normal development of the larva (Tung *et al.*, 1960a). Thus, while destined to form ectoderm, there are no substances unique to this layer that cannot be duplicated by other blastomeres.

Ooplasmic segregation

Since the work of Conklin (1932, 1933), it has been generally believed that ooplasmic segregation occurs in lancelets exactly as in ascidians (*e.g.*, Brien and Dalcq, 1948; Drach, 1948). Conklin maintained that "the localizations of materials in the Amphioxus egg are like those of ascidians, although not so sharply differentiated." Lacking the stages before the pronuclei meet, he found evidence for ooplasmic segregation in lancelets in the figures of Sobotta (1896) and van der Stricht (1897), although they made no such claims. In addition, Conklin (1933) was convinced from his own sections of eggs just before first cleavage that the mesodermal and chorda-neural crescents were distinguishable from endodermal and ectodermal areas; the mesodermal crescent was particularly visible because it consisted of more deeply staining cytoplasm. Curiously, Conklin did not mention the patchiness of yolk at the animal pole, although this was shown by Cerfontaine (1906).

Neither our results, nor those of Hirakow and Kajita (1990), have revealed any evidence for ooplasmic segregation in *Branchiostoma* such as occurs in ascidians. In lancelets, there are some small, localized aggregations of mitochondria in the animal cytoplasm, but no apparent segregation of mitochondria to the vegetal cytoplasm. The mitochondria associated with the zygote nucleus appear to be recruited by the migrating sperm nucleus and do not derive from the peripheral cytoplasm. Nowhere else in the fertilized lancelet egg is there a large concentration of mitochondria, comparable to that in the myoplasm of ascidian eggs. In addition, we found no differences in staining between regions destined to become the mesoderm, notochord, neural plate, or endoderm. As our Figures 8A, B, and 10A show, there is no crescent of more deeply staining cytoplasm anywhere in the egg. In the lancelet egg, the only type of dense granule is the yolk granule. The only regional difference in yolk is that it is scarcer in the animal hemisphere, destined for ectoderm and neural plate, than in the vegetal hemisphere, destined for mesoderm, endoderm, and notochord. It is very unlikely that the discrepancies between Conklin's conclusions and our finding are due to species differences. Although no TEM of fertilized eggs of *B. lanceolatum* has been done, yolk granules in the unfertilized egg are identical to those from *B. floridae* (Holland and Holland, 1991).

Conklin's interpretations of his own sections and the figures of others appear to have been chiefly based on his own preconceptions. In his ascidian paper (1905a), before obtaining any lancelet embryos, Conklin had concluded from van der Stricht (1896) that lancelet eggs must undergo ooplasmic segregation as in ascidians. Subsequently, Conklin interpreted all the evidence to support his ideas. First, he erroneously inferred from Sobotta (1897), who illustrated vesicles in the egg cortex, that the cortex, like that of ascidian eggs, contained not cortical granules, but numerous mitochondria. On the contrary, TEM has demonstrated that the cortex of lancelet eggs is packed with cortical granules and is nearly free of mitochondria (Holland and Holland, 1989a).

Next, Conklin was led further astray by errors of van der Stricht (1896), who thought that cortical granule disappearance resulted from their migration into the interior of the egg where they became the yolk-free patches in the animal cytoplasm. Conklin decided this was wrong in part; on disappearing, the peripheral cytoplasm migrated not into the interior of the egg, but to the vegetal pole and thus was the equivalent of the ascidian myoplasm. In truth, at fertilization, the cortical granules disappear because they undergo exocytosis and contribute to the formation of the fertilization envelope (Sobotta, 1897; Holland and Holland, 1989a). As further evidence of myoplasm, Conklin cited van der Stricht's figure 12, which, in fact, shows the yolk-free cytoplasm surrounding the vegetal pole plasm, which van der Stricht thought was the sperm remnant.

Finally, when interpreting his own sections, Conklin apparently saw things that simply weren't there. Publishing before it was common practice to photograph LM sections, Conklin (1933) drew "actual sections" (his text figure A) of fertilized *Branchiostoma* eggs showing the ectoplasm, the chorda-neural crescent, the endoderm, and the mesodermal crescent with its distinctive granules. This erroneous drawing has been reproduced in modern texts

(*e.g.*, Wickstead, 1975) and is the sole evidence for an ascidian-like ooplasmic segregation in lancelets.

Ooplasmic segregation and chordate phylogeny

Discussions about the phylogenetic origin of the chordates and the arrangements of the chordate subphyla are still highly contentious (*cf.* Ghiselin *et al.*, 1986; Jefferies, 1986; Erwin, 1991). The present study has demonstrated that a conspicuous, ascidian-style ooplasmic segregation does not occur in acraniates. Importantly, we have shown that such segregation is not a synapomorphy of ascidians and acraniates; instead it may be no more than an autapomorphy of ascidian tunicates, because conspicuous cytoplasmic rearrangements apparently do not occur in the fertilized egg in appendicularian tunicates (Holland *et al.*, 1988), which may be closest to the stem tunicate (Remane *et al.*, 1976; Holland, 1991).

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